Altered *hsrω* lncRNA levels in activated Ras background further enhance Ras activity in *Drosophila* eye and induces more R7 photoreceptors

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Abstract

We examined roles of *hsrω* lncRNAs in Ras signaling by down- or up-regulating them in *sev-GAL4* driven activated Ras^{V12} expressing *Drosophila* eye discs. Late pupal lethality and extra R7 photoreceptors in ommatidia caused by *sev-GAL4>*Ras^{V12} expression were significantly enhanced following simultaneous down- or up-regulation of *hsrω* lncRNAs. Ras signaling increased cell autonomously as well as non-autonomously, as revealed by enhanced p-MAPK expression, reduced Yan levels, greater RafRBDFLAG associated Ras and more R7 rhabdomeres. The further enhanced elevated Ras signaling seems to be due to post-transcriptional modifications in activities of Ras and its down-stream signaling members because of the disrupted intra-cellular dynamicity of many omega speckle associated hnRNPs and other RNA-binding proteins following down- or up-regulation of *hsrω* lncRNAs in elevated active Ras background. Co-altered *hsrω* RNA levels also modulated expression of certain sn/sno/scaRNAs and some other RNA processing genes affected in *sev-GAL4>*Ras^{V12}discs. Down-regulation of *hsrω* transcripts in such background elevated positive modulators of Ras signaling while *hsrω* up-regulation reduced negative-modulators, further enhancing Ras signaling in either conditions. Cell autonomous and non-autonomous enhancement of hyperactive Ras by lncRNAs has implications for cell signaling during normal and high Ras activity, commonly associated with some cancers.

Summary

Our findings highlight roles of *hsrω* lncRNAs in conditionally modulating the important Ras signaling pathway and provide evidence for cell non-autonomous Ras signaling in *Drosophila* eye discs.

Introduction

Evolution of multi-cellularity and the associated division of labour between different cell types has necessitated inter-cellular signaling pathways with complex network of regulatory circuits. The evolution of biological complexity has also been paralleled by substantial increase in the non-coding component in diverse genomes, and there is increasing realization in recent years that the large varieties of short and long non-coding RNAs (lncRNA) have great roles in cell signaling and gene regulation (Geisler and Coller, 2013; Huang et al., 2013; Jose, 2015; Lakhotia, 2016; Lakhotia, 2017a; Lakhotia, 2017b; Mattick and Makunin, 2006; Morris and Mattick, 2014; Peng et al., 2017). The lncRNAs like acal, mrhl, CRNDE, LncND, and URHC have been reported to regulate JNK, Wnt, insulin, Notch and ERK/MAPK signaling pathways, respectively (Arun et al., 2012; Ellis et al., 2014; Katsushima et al., 2016; Ríos-Barrera et al., 2015; Xu et al., 2014). Besides their roles in developmental regulation (Katsushima et al., 2016; Kotake et al., 2016; Lakhotia, 2017b; Misawa et al., 2017; Zhang et al., 2017a), diverse lncRNAs have been reported to interact with components of the various signaling pathways in cancer, activating or repressing their strength and thus affecting outcomes like cell proliferation or apoptosis (Lakhotia, 2016; Liu et al., 2015; Wang et al., 2015). An earlier study (Ray and Lakhotia, 1998) from our laboratory showed that mutant alleles of *ras* (ras^{E62K} and ras^{D38N}) dominantly enhanced the lethality due to nullisomic condition of the *hsrω* gene, which produces multiple lncRNAs (Lakhotia, 2011; Lakhotia, 2017a). The RAS/RAF/MAPK signaling pathway regulates many developmental pathways as it affects cell division, proliferation, growth as well as death, besides its major roles in many human cancers (Fernández-Medarde and Santos, 2011; Pylayeva-Gupta et al., 2011). Ectopic expression of activated Ras causes hyperplastic growth of the concerned organ in *Drosophila* as well (Karim and Rubin, 1998; Prober and Edgar, 2000).

In the present study, we further examined interaction between the *hsrω* gene and the Ras signaling pathway. Down- or up-regulation of *hsrω* lncRNAs, through *sev-GAL4* driven RNAi or its over-expression, respectively, exaggerated the phenotypes following ectopic expression of activated Ras producing *UAS-*Ras^{V12} transgene in developing eye discs, but had no effect on Ras signaling in eye discs with normal Ras activity. The *sev-GAL4* driven expression of Ras^{V12} is known to cause ommatidial derangement and rough eyes due to increase in number of R7 photoreceptor cells (Karim et al., 1996). Intriguingly, reduced as well as enhanced levels of *hsrω* lncRNAs in *sev-GAL4>*Ras^{V12} expressing eye discs enhanced Ras signaling due to substantial increase in levels of activated Ras and phosphorylated MAPK (pMAPK) in cell autonomous as well as non-autonomous manner. This resulted in further increase in number of the R7 photoreceptors. Activated Ras and its downstream component (RafRBDFLAG, Ras binding domain of Raf protein tagged with FLAG, (Freeman et al., 2010) were also present in cells that do not express the *sev-GAL4>UAS-*Ras^{V12} and *UAS-RafRBDFLAG* transgenes, indicating the possibility that activated Ras complex itself can move from the source cell to adjacent cells, leading to cell non-autonomous Ras signaling. Examination of transcriptomes of eye discs with down- or up-regulated *hsrω* transcripts in normal Ras or *sev-GAL4>*activated Ras expression

backgrounds and in eye discs expressing *sev-GAL4*>activated Ras in normal *hsrω* expression background revealed that either down- or up-regulation of *hsrω* lncRNAs in normal Ras background resulted in largely similar transcriptomic changes with some unique effects as well, but hardly any instances where specific genes showed opposite changes. Unexpectedly, levels of transcripts of none of the known major members of Ras/Raf/MAPK signaling pathway were significantly affected by altered *hsrω* RNA levels in eye discs with or without activated Ras expression background. Analysis of levels of expression of genes encoding transcription factors and RNA binding proteins revealed that down- or up-regulation of *hsrω* lncRNAs affected these two groups similarly or uniquely, but not oppositely. In addition, some members of the RNA processing machinery, several sn/snoRNAs and a scaRNA were up-regulated in activated Ras background but were down-regulated when the *hsrω* RNA levels were simultaneously lowered or over-expressed. Interestingly, while down-regulation of *hsrω* activity in activated Ras background resulted in up-regulation of some positive modulators of Ras signaling pathway, up-regulation of these transcripts caused down-regulation of several negative regulators of Ras/Raf/MAPK pathway. Consequently, the Ras activity increased in both cases. An over- or under-expression of the nuclear lncRNAs produced by the *hsrω* gene, affects dynamics of the omega speckle associated RNA binding proteins, including diverse hnRNPs (Lakhotia et al., 2012; Piccolo et al., 2018; Piccolo and Yamaguchi, 2017; Prasanth et al., 2000; Singh and Lakhotia, 2015). Bioinformatic analysis indicated that several of these known interactors of *hsrω* RNAs can bind to transcripts of modulators of Ras signaling, which may be responsible for the observed further elevation of Ras signaling following co-alteration in *hsrω* lncRNA levels in activated Ras expression background.

The present study thus shows that if levels of activated Ras are elevated for some reason in a cell, alterations in levels of lncRNAs, like those produced by *hsrω*, can further enhance Ras-signaling not only in those cells that express activated Ras but also non-autonomously in neighboring cells. These findings have implications for modulation of Ras signaling in disease conditions like cancer by lncRNAs functionally analogous to those produced by the *hsrω* gene. Initial results of this study were published as pre-print archive (Ray and Lakhotia, 2017).

Results

Down- as well as up-regulation of *hsrω* RNA levels aggravates phenotypes due to *sev-GAL4* driven expression of activated Ras in eye discs

We used *UAS-Ras*^{V12} transgene (Karim et al., 1996), which upon GAL4 driven expression produces mutant Ras that does not undergo RasGTP to RasGDP transformation and can be active even in absence of activation by the upstream receptor tyrosine kinase (RTK). The *sev-GAL4* driven ectopic expression of *UAS-Ras*^{V12} leads to additional R7 rhabdomeres and rough eye morphology (Karim et al., 1996). To alter levels of the >10kb nuclear lncRNAs produced by *hsrω* gene, the *UAS-hsrωRNAi* transgene was used to down-regulate the *hsrω* nuclear lncRNAs (Mallik and Lakhotia, 2009) while for up-regulating this gene, the GAL4 inducible *EP3037*

(Liao et al., 2000; Mallik and Lakhotia, 2009) allele was used. In addition, we also used another transgenic RNAi line, the *UAS-pUHEx2A* (R. Sahu and S. C. Lakhotia, unpublished), directed against the exon 2 of *hsrω* gene, and the *EP93D* over-expression allele of *hsrω* (Mallik and Lakhotia, 2009). In some experiments $hsr\omega^{66}$, which is a near null allele (Johnson et al., 2011) of *hsrω* gene, was also used to down-regulate *hsrω* RNA transcripts.

At 24±1°C, only 11-12% of *sev-GAL4> Ras^{V12}* (N =1058) expressing pupae eclosed as adult flies with rough eyes, besides having de-pigmented patches as well as occasional black spots in eyes (Fig 1A). Most of those failing to emerge died as pharates (Fig 1B). When either *hsrωRNAi* (N = 1177) or *EP3037* (N = 1109) was co-expressed in *sev-GAL4* driven Ras^{V12} background, none of the pupae eclosed, with a majority dying as early pupae (Fig. 1A, B), indicating that changes in *hsrω* transcripts enhanced the effects of ectopic expression of activated Ras. With a view to examine the adult eye phenotypes, the above three genotypes were grown at 18±1°C, since the strength of the GAL4 driven expression is reduced at lower temperatures (Brand et al., 1994; Mondal et al., 2007). When reared at 18±1°C, more than 80% flies (N = about 1000 flies for each genotype) eclosed in each case, with no early pupal lethality (Fig. 1C). In this case also, *sev-GAL4* driven activated Ras expression caused roughening of eyes (Fig. 1E, J) compared to normal eyes in *sev-GAL4>GFP* flies (Fig. 1D, I). Interestingly, those co-expressing *sev-GAL4* driven *hsrωRNAi* or *EP3037* and Ras^{V12} showed greater roughening of eyes and ommatidial fusion than in *sev-GAL4>Ras^{V12}* eyes (Fig. 1F, G, K, L).

When *UAS-pUHEx2A* was expressed along with activated Ras under *sev-GAL4* at 24±1°C, early pupal death similar to that in case of *UAS-hsrωRNAi* was observed (data not shown). Similarly, over expression of hsrω RNAs due to *EP93D* allele under the *sev-GAL4* driver too resulted in pupal lethality (data not shown) similar to that noted above for *sev-GAL4>Ras^{V12} EP3037* genotype. The *sev-GAL4* driven activated Ras expression in the $hsr\omega^{66}$, reared at 24±1°C, also resulted in greater roughening of eyes and fusion of ommatidia in all (N = 500) emerging adult flies (Fig. 1H, M). However, enhanced or early pupal death was not found in this case. The absence of enhanced pupal death in *sev-GAL4>Ras^{V12} $hsr\omega^{66}$* individuals, unlike that seen in the *sev-GAL4>Ras^{V12} UAS-hsrωRNAi* or *sev-GAL4>Ras^{V12} EP3037* genotypes may be related to the fact that all cells in eye discs in *sev-GAL4>Ras^{V12} $hsr\omega^{66}$* carry greatly reduced levels of *hsrω* transcripts unlike the other genotypes in which only the cells expressing *sev-GAL4* have reduced or elevated *hsrω* transcripts. As discussed elsewhere (Ray and Lakhotia, 2016), the imbalance between the *sev-GAL4* expressing and rest of the eye disc cells in the latter two genotypes leads to Dilp8 secretion and consequent pupal death.

In view of the similar results with two different *hsrωRNAi* transgenes, two different *EP* alleles and a near null allele of *hsrω*, in most of the subsequent studies we used the *UAS-hsrωRNAi* and *EP3037* to down- or up-regulate *hsrω* transcript levels, respectively.

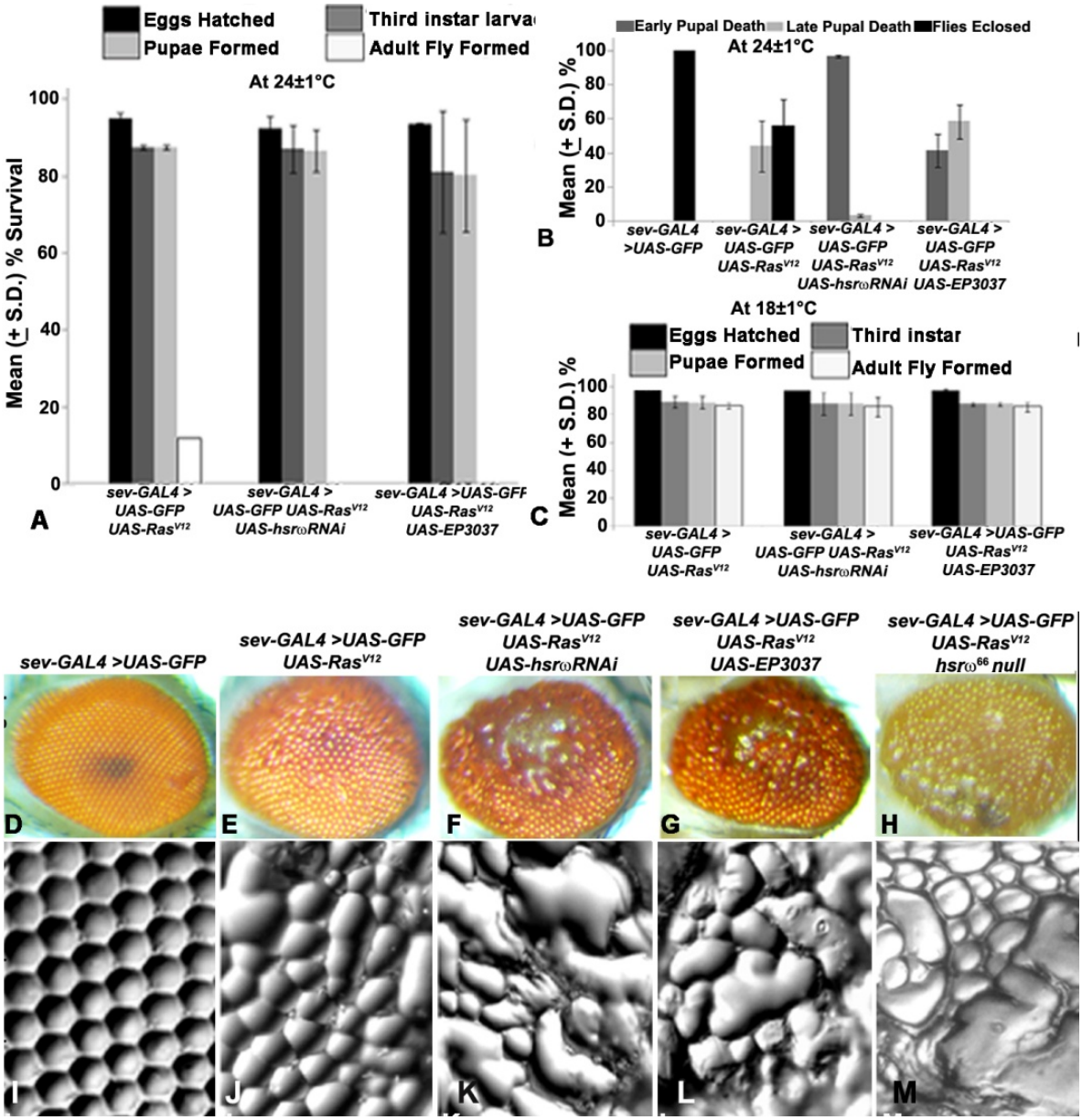

Fig. 1. Alterations in levels of *hsrω* RNAs enhance the pupal lethality and roughening of eyes due to *sev-GAL4* driven expression of activated Ras. A-C Histograms showing mean percent (± S.D.) survival (Y-axis) of different genotypes (X-axis) till different stages of development (**A**) and mean percent survival of pupae when reared at 24±1°C (**B**) and mean percent (± S.D.) survival (Y-axis) of different genotypes (X-axis) till different stages of development when reared at 18±1°C (**C**), **D-H** Photomicrographs and **I-M** nail polish imprints of adult eyes of individuals of different genotypes, noted above each column, reared at 18±1°C (**D-G** and **I-L**) or at 24±1°C (**H** and **M**).

The numbers of photoreceptor cells in developing ommatidia in eye discs of different genotypes was counted. Since the third instar larval eye disc contains ommatidia at different stages of

development, ommatidia from the posterior most region that could be separately demarcated were examined from five representative eye discs for each genotype. The high degree of derangement and fusion of ommatidia in *sev-GAL4>Ras^{V12} UAS-hsrωRNAi* and *sev-GAL4>Ras^{V12} EP3037* genotypes reduced the probability of finding individual un-fused ommatidia and, therefore, the total number that could be examined in these cases was rather low. The photoreceptor cells were recognized on the basis of Elav expression while the *sev-GAL4* expressing cells were recognized on the basis of GFP expression. When third instar larval eye discs of *sev-GAL4>Ras^{V12}* or *sev-GAL4>Ras^{V12} UAS-hsrωRNAi* or *sev-GAL4>Ras^{V12} EP3037* genotypes, grown at 24±1°C, were immunostained with anti-Elav to mark the neuronal photoreceptor cells in eye discs, it was evident that both down- or up-regulation of *hsrω* transcripts in *sev-GAL4* driven activated Ras expression background further enhanced the number of photoreceptor cells and consequently the ommatidial disarray (compare Fig 2A-B with Fig 2C-E). In agreement with the reported expression of *sev-GAL4* driver (Ray and Lakhotia, 2015), the *sev-GAL4* directed *UAS-GFP* transgene, present in all these genotypes, was expressed in a subset of photoreceptor cells, and in the two future cone cells. Accordingly, the average number of GFP+ve and Elav+ve cells (photoreceptor cells with the Sevenless expression) in each *sev-GAL4>UAS-GFP* ommatidium varied between 3-4, that of GFP+ve and Elav-ve (future cone cells) between 1-2 cells while the GFP-ve and Elav+ve rhabdomeres (photoreceptor cells without the sevenless expression) varied between 3-4. Identification of these three classes of cells in third instar larval eye discs from *sev-GAL4>UAS-Ras^{V12}* larvae showed a small but specific increase only in GFP+ve and Elav+ve cells (Fig 2K), i.e., *sev-GAL4* expressing photoreceptor cells. Interestingly, the numbers of GFP+ve and Elav+ve cells in eye discs that had altered levels of *hsrω* RNAs in *sev-GAL4>UAS-Ras^{V12}* background, showed a much greater increase (Fig. 2K), with the most pronounced increase in *sev-GAL4>UAS-Ras^{V12} UAS-hsrωRNAi* genotype (Fig 2C, K).

It may be noted that, in agreement with an earlier report (Mallik and Lakhotia, 2011), *sev-GAL4* driven expression of *UAS-hsrωRNAi* or *EP3037* in normal wild type Ras expression background did not cause any roughening of eyes (not shown).

These results clearly showed that *sev-GAL4* driven alterations in levels of *hsrω* transcripts in activated Ras expression background enhanced the number of *sev-GAL4* expressing neuronal cells but not of the *sev-GAL4* expressing non-neuronal future cone cells.

The additional photoreceptors in eye discs with altered levels of *hsrω* transcripts in activated Ras expression background are R7 type

The Ras/Raf/MAPK signaling dependent differentiation of R7 photoreceptor, the last one of the 8 photoreceptors to differentiate, is initiated by binding of the Boss ligand to the Sevenless receptor tyrosine kinase (RTK) (Mavromatakis and Tomlinson, 2016; Tomlinson and Struhl, 2001). Of the multiple R7 precursor cells, only the one cell in which the Boss ligand binds with and activates the RTK, which in turn activates the downstream Ras by converting the GDP-

bound Ras to active GTP-bound Ras, differentiates into R7 in normal development. Activation of Ras initiates signaling cascade involving a series of phosphorylation reactions culminating in phosphorylation of MAPK, which translocates to nucleus and triggers the downstream events for R7 differentiation (Karin and Hunter, 1995). Since the Ras^{V12} does not need ligand binding for activation, the *sev-GAL4>UAS-Ras*^{V12} expression directly drives differentiation of two or more R7 photoreceptor cells per ommatidium.

Eye discs from larvae of different genotypes were immunostained with Runt antibody to confirm that the additional GFP+ve and Elav+ve photoreceptor cells seen in the experimental genotypes indeed belonged to the R7 lineage. In wild type third instar larval eye discs, each developing ommatidium shows two Runt-positive photoreceptors, viz. R7 and R8 (Edwards and Meinertzhagen, 2009; Tomlinson et al., 2011). Since the R7 and R8 photoreceptors are present one above the other in each ommatidium, a given optical section shows either the R7 or R8, which could be further distinguished because the *sev-GAL4>UAS-GFP* is expressed only in R7. In wild type discs, R7 cells formed a well arranged pattern with only one Runt and GFP-positive rhabdomere in each developing ommatidium (Fig 2F). Most of the ommatidia in eye discs expressing *sev-GAL4* driven activated Ras showed two Runt and GFP-positive R7 photoreceptor cells (Fig 2G), resulting in derangement of the regular pattern. Down-regulation of *hsrω* RNA in the same background led to about four R7 photoreceptors in each ommatidium with severely disarrayed ommatidial pattern (Fig 2H, L). Up-regulation of *hsrω* RNA through *EP3037* expression in activated Ras background also increased the number of R7 photoreceptors to about three per ommatidium (Fig 2I, L), which is less than that in *sev-GAL4>UAS-Ras*^{V12} *UAS-hsrωRNAi* eye discs. Down-regulating *hsrω* RNA level using the *hsrω*^{66} allele too, resulted in derangement of photoreceptor array in developing eye discs (Fig 2E) due to increase in neuronal cells (Fig 2K), which were confirmed by Runt staining to be R7 photoreceptors (Fig 2J, L).

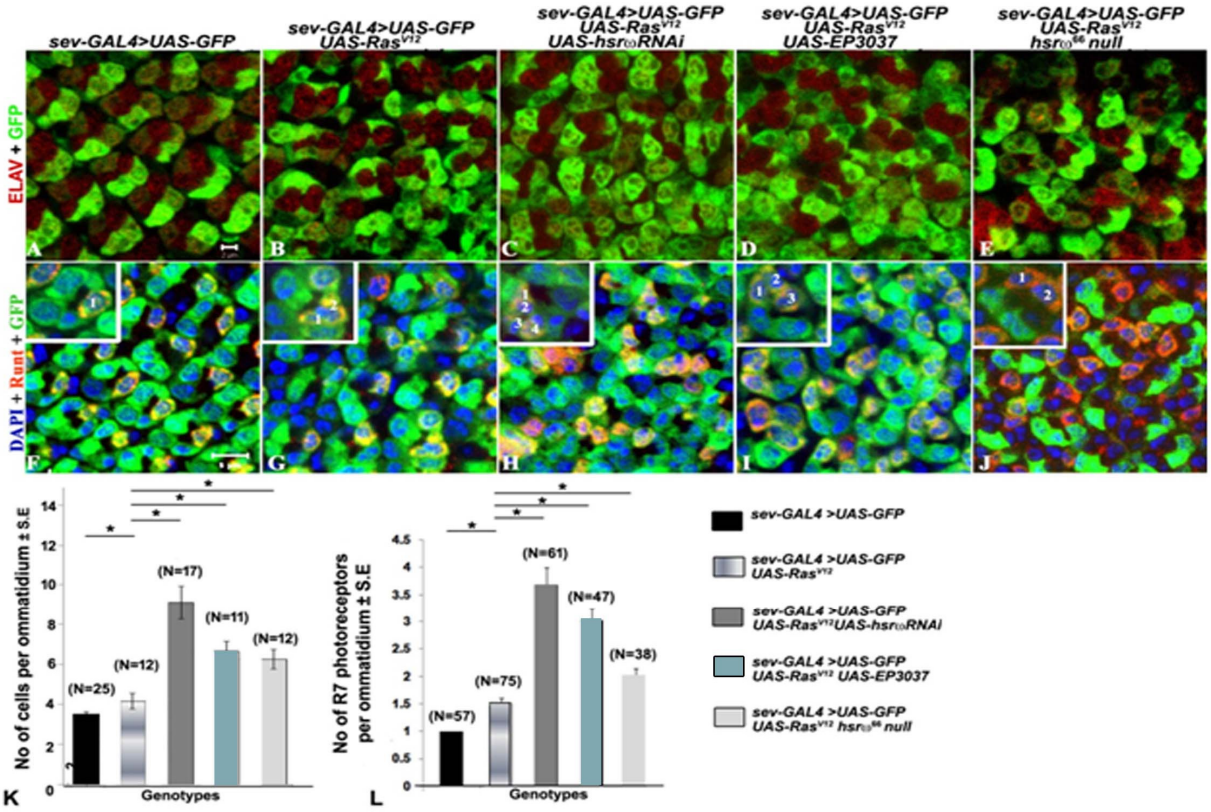

Fig. 2. Changes in *hsrω* RNA levels in activated Ras expressing background promotes recruitment of more cells to R7 photoreceptor/neuronal fate. A-J Confocal optical sections *of third instar larval eye discs of different genotypes (noted above each column) showing photoreceptor cells in developing ommatidia marked by Elav staining (red, **A-E**) and the Runt positive R7 photoreceptors (red, **F-J**); the *sev-GAL4>GFP* expressing cells are green in **A-J**; counterstaining with DAPI (blue, **F-J**). Insets in **F-J** show single representative ommatidium, with the Runt and GFP+ve cells numbered. Scale bar denotes 2µm in **A** and 5µm in **F** and applies to **A-E** and **F-J**, respectively. **K-L** Histograms showing mean number (± S.E., Y-axis) of neuronal (Elav +ve) cells per ommatidium in different genotypes (**K**) and mean number of R7 photoreceptor cells (Runt and GFP +ve) per ommatidium (**L**) in third instar larval eye discs in different genotypes (X-axis) identified in the key on right. The number of ommatidia (N) examined for data in **K** and **L** are noted in parentheses above each histogram bar. A horizontal line connecting specific pairs of histogram bars and the * mark indicates that the given pair of means are significantly different ($P \leq 0.05$) on Student's *t*-test.

Altered *hsrω* RNA levels further enhance Ras signaling in cell autonomous as well as non-autonomous manner in eye discs expressing *sev-GAL4* driven activated Ras

The above noted increase in number of R7 photoreceptors in each ommatidium in eye discs with altered levels of *hsrω* transcripts in activated Ras expression background suggested further increase in Ras signaling. In order to measure the level of Ras signaling, we examined

distribution of p-MAPK since phosphorylation of MAPK and its nuclear translocation is a measure of active Ras signaling (Karin and Hunter, 1995). In addition, we also examined levels of Yan, a transcription factor which is negatively regulated by Ras signaling (Brunner et al., 1994; O'Neill et al., 1994).

In normally developing control eye discs (*sev-GAL4>UAS-GFP*), only a few cells in each ommatidium showed nuclear p-MAPK localization (Fig 3A, M). Expression of activated Ras led to greater number of cells showing nuclear p-MAPK staining (Fig 3B, M) besides an overall increase in p-MAPK presence. When *hsrω* RNA levels were either down- (Fig 3C) or up-regulated (Fig 3D), the number of cells with nuclear p-MAPK showed a steep increase (Fig 3C-D, M) with concomitant rise in overall p-MAPK levels in the eye discs. Interestingly, not only the cells expressing *sev-GAL4* driver, identified by the *UAS-GFP* expression, but non *sev-GAL4* expressing GFP-negative cells (marked by arrows in Fig 3B-D) also showed higher p-MAPK levels. This suggested a non-autonomous increase in Ras signaling.

In control *sev-GAL4>UAS-GFP* third instar larvae, the Yan transcription factor is expressed all over the eye disc posterior to the morphogenetic furrow (MF), with especially stronger presence in the MF itself (marked by arrows in Fig 3E-H). The Yan staining progressively declined as one moved from anterior to posterior region of eye discs, where its presence in the differentiated photoreceptors was sparse. Following *sev-GAL4* driven expression of activated Ras a small but perceptible decrease in Yan expression along the antero-posterior axis of the discs and at the MF was noted (Fig 3F). Either down- or up-regulation of *hsrω* RNA levels in the *sev-GAL4* driven activated Ras expression background further reduced the expression of Yan in the eye discs (Fig 3G-H), including in the MF cells. Since the *sev-GAL4* driver has no expression at the MF (Ray and Lakhotia, 2015), the distinct reduction in Yan staining all over the eye disc, including the MF, is clearly indicative of a cell non-autonomous Ras signaling, which was further enhanced when levels of *hsrω* non-coding transcripts were reduced or elevated.

With a view to ascertain if the above changes that suggested elevated Ras signaling in the test genotypes were associated with enhanced Ras expression and/or with a higher proportion of Ras being in an active form, we co-immunostained developing eye discs of wandering third instar control larvae of different genotypes for Ras and RafRBDFLAG since the *UAS-RafRDBFLAG* construct (Freeman et al., 2010), which expresses the FLAG-tagged active Ras binding domain of Raf, binds only with active Ras. Fig. 3I-L present confocal images of immunostained eye discs while the Fig. 3N-O show results of quantification of mean intensity levels of Ras, RafRBDFLAG and co-localization of Ras and RafRBDFLAG, respectively, to provide an estimate of active Ras levels in eye discs of different genotypes.

Following expression of *sev-GAL4* driven *UAS-RafRBDFLAG* in developing eye discs of wandering third instar control larvae with normal developmental Ras expression, little co-localization of the RafRBDFLAG with the native Ras was detectable (Fig 3I). In contrast, FLAG tagged RafRBD was substantially co-localized with the Ras in eye discs expressing *sev-GAL4*

driven activated Ras, indicating presence of Ras in the active form (Fig 3J). Down- or up-regulation of *hsrω* RNAs in this background clearly enhanced the number of cells that showed distinctly co-localized RafRBDFLAG and Ras (Fig 3K, L). Interestingly, as may be noted from Fig 3J-L, eye discs co-expressing *sev-GAL4* driven *UAS-Ras*^{V12} and *UAS-hsrωRNAi* or *EP3037* had a greater number of GFP-ve cells, adjoining the GFP+ve cell, which also showed colocalized Ras and RafRBDFLAG. Since neither RafRBDFLAG nor activated Ras was expressed in the non *sev-GAL4* expressing GFP-ve cells, their co-localization in such cells suggests movement of activated Ras complex from *sev-GAL4* expressing cells to the neighboring cells.

With a view to know if the increased colocalization seen in more cells in eye discs expressing activated Ras without or with altered *hsrω* RNA levels reflected equal or differential elevation in levels of total Ras, activated Ras and RafRBDFLAG proteins, we used the histo option of the Zeiss Meta 510 Zen software to quantify the Ras, FLAG, DAPI and GFP fluorescence signals in these genotypes. For this purpose maximum projection images of 13 optical sections which showed Elav positive photoreceptor cells of each eye disc of different genotypes (N for each genotype is noted in Fig. 3N) were used to quantify the respective fluorescence intensities. As seen in Fig. 3N, the total Ras is expectedly higher in *sev-GAL4>UAS-Ras*^{V12} than in *sev-GAL4>UAS-GFP* eye discs. More than 2 times further increase in Ras staining in discs co-expressing *UAS-hsrωRNAi* or *EP3037* with activated Ras, clearly shows that the under- or over-expression of hsrω transcripts in activated Ras over-expression background further enhanced Ras levels. The increase in GFP staining in *sev-GAL4>UAS-Ras*^{V12} *UAS-hsrωRNAi* correlates with the earlier noted greater increase in *sev-GAL4* driven GFP expressing cells in these eye discs. The more or less comparable levels of DAPI fluorescence in the samples of eyes discs in different genotypes indicates that the increase in Ras or GFP activity in specific genotypes is not due to a general increase in number of cells in some genotypes. It is significant that the levels of RafRBDFLAG protein showed the expected increase in *sev-GAL4>UAS-Ras*^{V12} over that in *sev-GAL4>UAS-GFP* eye discs but co-expression of *UAS-hsrωRNAi* or *EP3037* with activated Ras was not associated with further increase in the FLAG staining (Fig. 3N).

In order to determine how much of the enhanced levels of Ras in *sev-GAL4>UAS-Ras*^{V12} and more so in *sev-GAL4>UAS-Ras*^{V12} *UAS-hsrωRNAi* and *sev-GAL4>UAS-Ras*^{V12} *EP3037* eye discs was in activated form, we examined colocalization of Ras and RafRBDFLAG fluorescence signals (Fig. 3O) using the same sets of the maximum projection images of eye discs used for quantification of Ras and RafRBDFLAG in different genotypes (Fig. 3N). The co-localization option of the Zeiss Meta 510 Zen software was used as described in the Zeiss manual (https://www.zeiss.com/content/dam/Microscopy/Downloads/Pdf/FAQs/zenaim_colocalization.pdf). In agreement with the ectopic expression of activated Ras in *sev-GAL4>UAS-Ras*^{V12} eye discs, nearly 40% of RafRBDFLAG was associated with Ras compared to only about 5% of the FLAG signal being associated with Ras in *sev-GAL4>UAS-GFP* discs. Interestingly, co-expression of *UAS-hsrωRNAi* or *EP3037* in *sev-GAL4>UAS-Ras*^{V12} eye discs resulted in further

increase in association of Ras and RafRBDFLAG proteins (Fig. 3O), indicating that a greater proportion of Ras in these cells is in activated form with which the RafRBDFLAG can bind.

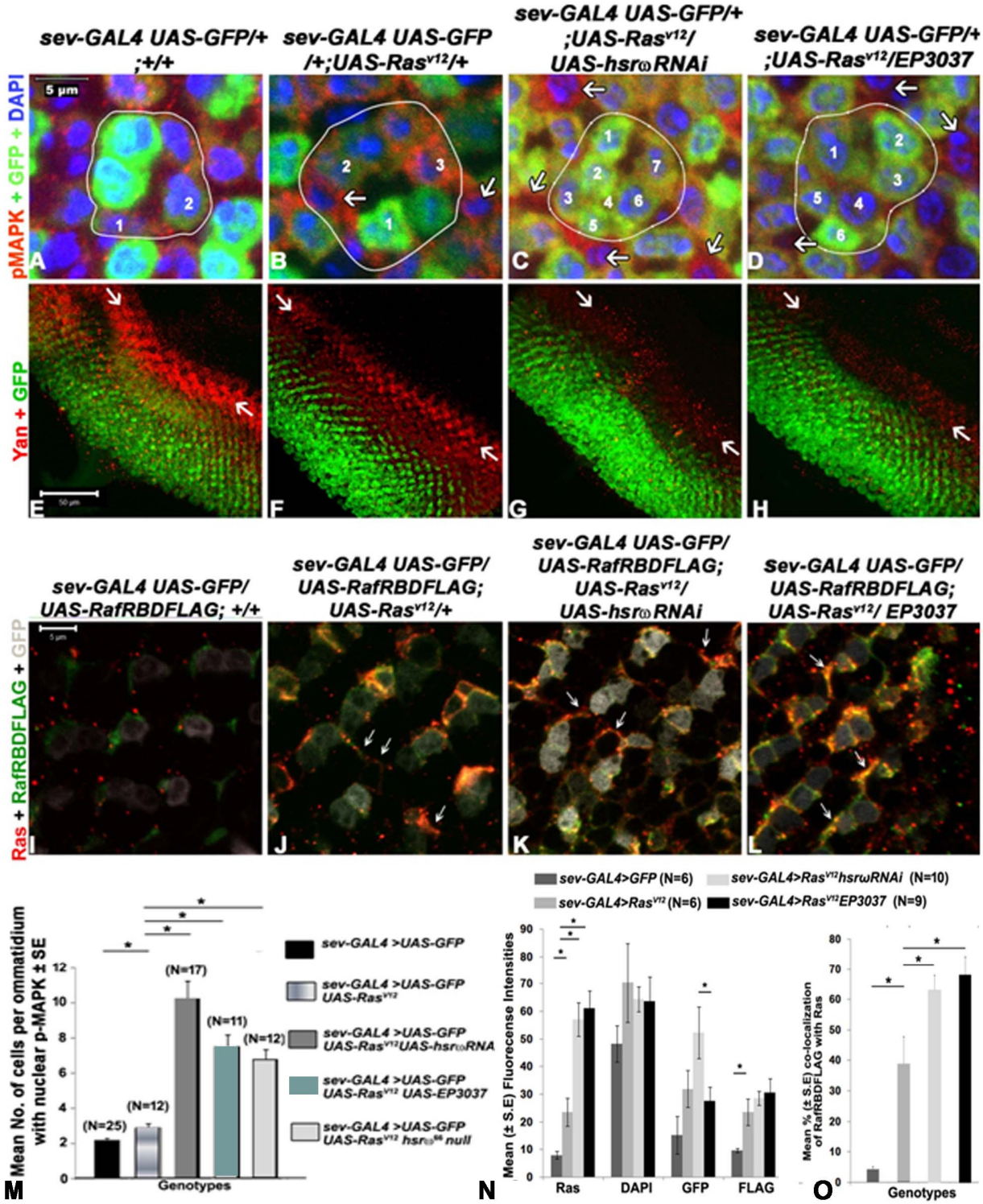

Fig. 3. Alterations in *hsrω* RNA levels in activated Ras expression background leads to increase in cell autonomous as well as non- autonomous Ras signaling. A-D Confocal optical sections of parts of third instar larval eye discs of different genotypes (noted above each column) immunostained with p-MAPK antibody (red); *sev-GAL4* driven GFP expressing cells are green (counterstained with DAPI, blue); a representative developing ommatidium in each is demarcated by white line with the rhabdomeres with nuclear p-MAPK being numbered. Some of the non- GFP expressing cells but with distinct p-MAPK staining in **B-D** are indicated by white arrows. Scale bar in **A** denotes 5µm and applies to **A-D. E-H** Confocal optical sections of third instar larval eye discs of different genotypes (noted above each column) showing immunostaining for Yan (red) and *sev-GAL4* driven GFP fluorescence (green). The Morphogenetic furrow is indicated by white arrows. Scale bar in **E** denotes 50µm and applies to **E-H. I-L** Confocal optical sections of parts of third instar larval eye discs of different genotypes (noted above each column) immunostained with Ras (red) and FLAG (green) antibodies, the *sev-GAL4>GFP* expressing cells are grey. Scale bar in **I** denotes 5µm and applies to **I-L. M** Histogram showing mean number of cells with nuclear p-MAPK per ommatidium in third instar larval eye discs in different genotypes (X-axis), identified in the key on right. The number of ommatidia (N) examined for data in **M** are noted in parentheses above each histogram bar. **N** Histogram showing intensities (Y-axis) of Ras, DAPI, GFP and RafRBDFLAG fluorescence (X-axis) in third instar larval eye discs of different genotypes (key above). **O** Colocalization of RafRBDFLAG and Ras, expressed as mean percent (± S.E) of RafRBDFLAG protein associated with Ras, to compare relative levels of activated Ras in different genotypes. Number of eye discs observed (N) is given in parenthesis for each genotype in the key above **N** and **O** panels. A horizontal line connecting specific pairs of histogram bars and the * mark indicate that the given pair of means are significantly different ($P \leq 0.05$) on Student's *t*-test.

In order to see if the above noted increase in Ras and activated Ras levels was associated with increased transcription of the resident *Ras* and/or *UAS-Ras^{V12}* transgene, we examined the levels of Ras transcripts derived from these two sources using semi-quantitative RT-PCR with primers designed to differentiate between these two transcripts. The normal resident *Ras* gene transcripts remained more or less comparable in all the four genotypes (*sev-GAL4>UAS-GFP*, *sev-GAL4>UAS-Ras^{V12}*, *sev-GAL4>UAS-Ras^{V12} hsrωRNAi* and *sev-GAL4>UAS-Ras^{V12} EP3037*) and likewise, the transcripts derived from the *Ras^{V12}* transgene remained similar in *sev-GAL4>UAS-Ras^{V12}* and those co-expressing *hsrωRNAi* or *EP3037* with *UAS-Ras^{V12}* (Supplementary Fig S1 A and B). This indicated that the elevated Ras activity in the latter two genotypes with altered *hsrω* RNA levels is unlikely to be due to increased transcription of *UAS-Ras^{V12}* transgene or the resident *Ras* gene. As noted later, the RNA-seq data also did not show any significant increase in Ras transcripts in *sev-GAL4>UAS-Ras^{V12}* eye discs co-expressing *hsrωRNAi* or *EP3037* compared to those expressing only activated Ras.

The *sev-GAL4* driven increase or decrease in *hsrω* lncRNA levels in normal Ras background had largely similar effects on the transcriptome of third instar larval eye discs

The observed elevation in Ras signaling by down- as well as up-regulation of *hsrω* transcripts in *sev-GAL4>UAS-Ras*^{V12} expression background was unexpected. With a view to understand the underlying reasons for such a common effect, we undertook sequencing of total RNAs from eye discs of *sev-GAL4>hsrωRNAi, sev-GAL4>EP3037*, *sev-GALl4>UAS-Ras*^{V12}, *sev-GAL4>UAS-Ras*^{V12} *hsrωRNAi* and *sev-GAL4>UAS-Ras*^{V12} *EP3037* larvae.

Analysis of RNA-seq data revealed that a large proportion of transcripts were indeed similarly affected by down- or up-regulation of *hsrω* RNA in normal as well as activated Ras expression background (Fig. 4; Supplementary Table S3, Sheets 1 and 2). A comparison of transcriptomes of *sev-GAL4>hsrωRNAi* and *sev-GAL4>EP3037* eye discs revealed that in each case the number of genes down regulated was more (Fig. 4A and B) than those up-regulated. Interestingly, while 319 genes were commonly down regulated (Fig. 4A) and 15 were commonly up-regulated (Fig. 4B) in the two genotypes, only 2 genes showed opposing trends between *sev-GAL4>UAS-hsrωRNAi* and *sev-GAL4>EP3037* eye discs (Fig 4 C-D). While a detailed analysis of the transcriptomic changes in these two genotypes would be considered elsewhere, a brief analysis of the commonly affected genes that appear to be relevant in the present context is presented here.

Gene Ontology analysis of the 319 commonly down regulated genes identified several R7 photoreceptor differentiation and Ras signaling cascade genes. Most of the Ras signaling regulation genes were part of Ras/Rho signaling pathway (*Rhogef64c, CG43658, CG5937, dab*) while others were negative regulators of Ras signaling (*Nfat* and *Klu*). The R7 differentiation pathway genes mainly acted upstream of Ras protein but downstream of receptor tyrosine kinase (*drk*, *sos*) (Olivier et al., 1993). Changes in activities of these genes apparently did not significantly alter the Ras signaling and R7 differentiation pathway since as noted above, *sev-GAL4* driven expression of *hsrωRNAi* or of *EP3037* did not affect ommatidial differentiation.

We also checked the status of known transcription factors (Rhee et al., 2014) and RNA binding proteins (RNA binding protein Database at http://rbpdb.ccbr.utoronto.ca) in *Drosophila melanogaster* as hsrω transcripts are known to bind to various RNA binding proteins regulating gene expression and RNA processing (Lakhotia et al., 2012; Piccolo et al., 2018; Piccolo and Yamaguchi, 2017; Prasanth et al., 2000; Singh and Lakhotia, 2015). Out of the nearly 1000 known transcription factors (Rhee et al., 2014), 91 were commonly down-regulated following down- or up-regulation of *hsrω* transcripts (Fig. 4E) while 4 and 74 were uniquely down regulated in *sev-GAL4>UAS-hsrωRNAi* and *sev-GAL4>EP3037* eye discs, respectively (Fig. 4E). Among the 259 known RNA binding proteins (RBP), 14 were commonly down regulated in either of the genotypes that alter the *hsrω* RNA levels, while 16 of these were significantly down regulated only in *sev-GAL4>UAS-EP3037* eye discs while 2 were significantly down regulated only in *sev-GAL4>UAS-hsrωRNAi* (Fig. 4F). Interestingly, as shown later (Fig. 8A), all of the 16 RBPs, which showed significant down regulation in *sev-GAL4>UAS-EP3037*, also showed a downward trend in *sev-GAL4>UAS-hsrωRNAi* eye discs. These 16 RBPs included some of the *hsrω* lncRNAs interactors like Hrb87F, Caz/dFus, TDP-43 (dTBPH) (Lakhotia et al., 2012;

Piccolo et al., 2018; Piccolo and Yamaguchi, 2017; Prasanth et al., 2000; Singh and Lakhotia, 2015). Surprisingly, none of the examined RBPs were up-regulated when *hsrω* transcripts were altered.

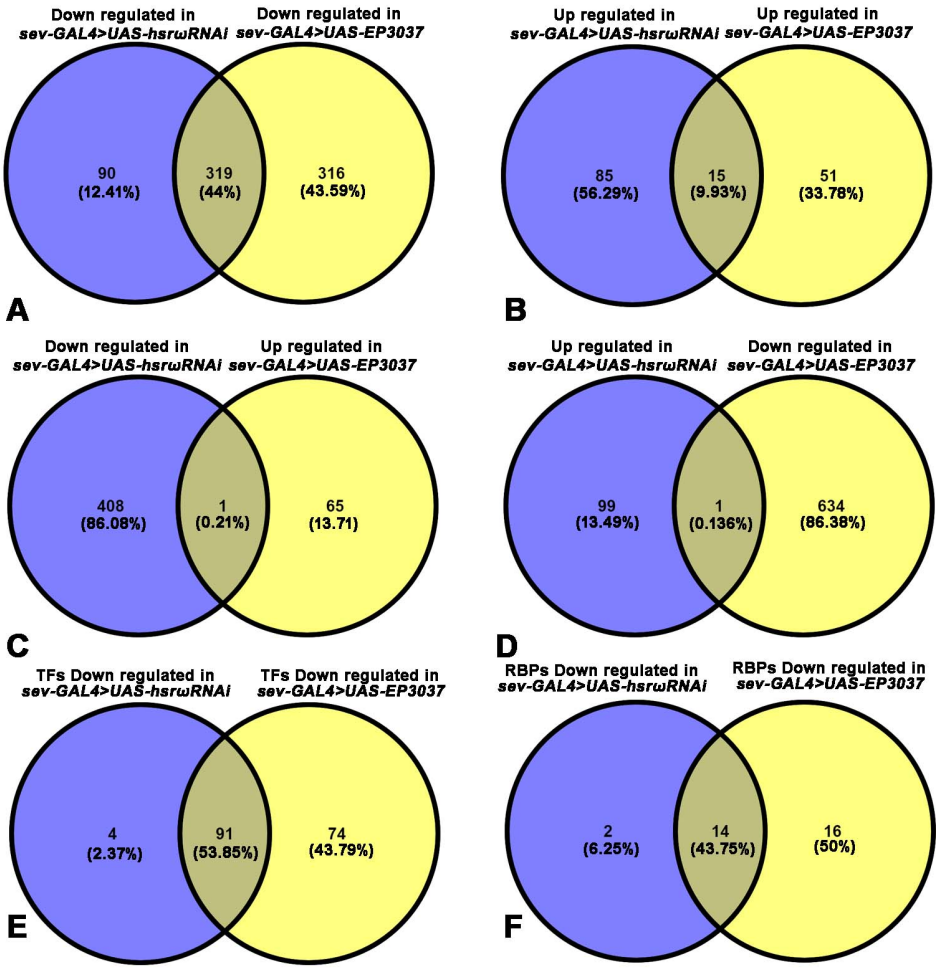

Fig. 4. The *sev-GAL4* driven down or up regulation of *hsrω* RNA in normal Ras expression background caused largely common transcriptomic changes. A-B Venn diagrams showing numbers of genes down- (**A**) or up-regulated (**B**) in third instar *sev-GAL4>UAS-hsrωRNAi* or *sev-GAL4>EP3037* larval eye discs when compared with *sev-GAL4>UAS-GFP* control eye discs. **C-D** Venn diagrams showing number of genes down-regulated in *sev-GAL4>UAS-hsrωRNAi* but up-regulated in *sev-GAL4>UAS-EP3037* eye discs (**C**) and vise-versa (**D**). **E-F** Venn diagrams showing numbers of genes encoding transcription factors (**E**) and RNA binding proteins (**F**) that were commonly or uniquely down-regulated in the two genotypes.

Down- or up-regulation of *hsrω* transcripts in activated Ras background commonly affected many genes including RNA processing components

The *sev-GAL4>UAS-Ras*^{V12} eye discs showed differential expression of many genes, with 374 genes being down-regulated and 138 up-regulated, when compared with *sev-GAL4>UAS-GFP* eye discs (List 1 in Fig 5A and B; Supplementary Table S3, Sheet 3). Besides the expected increase in levels of transcripts of genes involved in cell growth, proliferation and differentiation, transcripts of many genes involved in RNA biosynthesis, metabolism and processing were also up-regulated when compared with *sev-GAL4>UAS-GFP* eye discs. As expected, the levels of Ras transcripts were significantly higher in *sev-GAL4>UAS-Ras*^{V12} than in *sev-GAL4>UAS-GFP*. In agreement with the above noted RT-PCR results (Supplementary Fig. S1A, B), the RNA seq data also showed that levels of Ras transcripts did not show any further significant increase when *hsrω* RNA levels were down- or up-regulated in *sev-GAL4* driven activated Ras expression background. The RNA-seq data also revealed that levels of transcripts of the genes that act directly downstream in the Ras signaling cascade were not further up-regulated in eye discs co-expressing *sev-GAL4* driven *UAS-Ras*^{V12} and *hsrωRNAi* or *EP3037*.

Eye discs with *sev-GAL4* driven activated Ras expression showed the expected up-regulation of several genes involved in R7 cell differentiation (e.g., *salm, ten-m, cadn, svp, dab, nej*) and also of certain genes involved in photoreceptor development (e.g., *rno, doa, pdz-gef, jeb, atx2* and *csn4*). However, none of these genes showed any further change when *hsrωRNAi* or *EP3037* was co-expressed with activated Ras, except for *svp*, whose transcripts were further up-regulated in eye discs co-expressing *sev-GAL4* driven activated Ras and *hsrωRNAi* but not in those co-expressing activated Ras and *EP3037* (also see later). The RNA seq data for several of the Ras signaling cascade genes were validated through a real-time qRT-PCR analysis in different genotypes (Supplementary Fig. S1C). In all cases, results of RNA-seq and qRT-PCR were concordant.

In order to understand the basis for the unexpected similar enhancing effect of down- or up-regulation of *hsrω* transcripts on the Ras signaling in eye discs that were expressing activated Ras under the *sev-GAL4* driver (Supplementary Table S3, Sheets 4, 5), we first examined genes that were commonly down- or up- regulated following expression of *hsrωRNAi* or *EP3037* in *sev-GAL4* driven activated Ras expression background (encircled in red and white, respectively, in Fig. 5A and B). The group that was down-regulated by activated Ras expression and further down-regulated when *hsrωRNAi* or *EP3037* was co-expressed included only one gene (encircled in red in Fig 5A), while the group that was up-regulated following activated Ras expression and further up-regulated when *hsrω* RNA levels were reduced or elevated included three genes (encircled in red in Fig. 5B). The single gene in the first group, *CG13900* (*Sf3b3*), codes for a splicing factor 3b subunit involved in mRNA splicing. It remains to be seen if it modulates post-transcriptional processing of other transcripts, including of those in the Ras pathway. The three genes up regulated on Ras activation and further up-regulated on co-alterations in *hsrω* RNA levels were *CG15630*, *Hsp70Bb* and *CG14516*. On the basis of their described roles in the Flybase, however, none of these three genes appeared to be directly involved in modulating Ras pathway.

Among the genes that showed differential changes upon down or up-regulation of the *hsrω* transcripts in activated Ras expressing eye discs, was a group involved in ribosome biogenesis (*CG7275, CG7637, CG14210, hoip, bka, CG11920, CG9344, nhp2, CG11563, mrpl20, CG7006*) that was down regulated in activated Ras expressing discs with down-regulated *hsrω* transcripts. Expression of these genes was, however, not affected when *EP3037* was co-expressed with activated Ras.

A total of 88 (9.3%) genes that were up-regulated in *sevGAL4>Ras*^{V12}, when compared with *sevGAL4>UAS-GFP* control eye discs, were commonly down-regulated in *sevGAL4>Ras*^{V12} *hsrωRNAi* or *sevGAL4>Ras*^{V12} *EP3037* eye discs compared with *sevGAL4>Ras*^{V12} (encircled in white in Fig. 5A, and C-D). Many of the commonly down-regulated 88 genes (Fig. 5C) belonged to diverse GO terms without any apparent and known association with Ras signaling. However, one of these genes, the *dlc90F*, encoding a dynein light chain protein is reported to bind to Raf (Friedman et al., 2011) and to be a positive regulator of FGF signaling (Zhu et al., 2005). Not much is known about its role in Ras signaling. Rest of these 88 genes belonged to transcription factor (TF), or RNA-binding protein (RBP) families or those encoding snoRNA/scaRNA. The TF and RBP genes are considered later (Fig. 8). The commonly down-regulated snoRNAs and scaRNA genes (Fig. 5D) were significantly up-regulated by activated Ras expression but co-expression of either *hsrωRNAi* or *EP3037* led to their significant down-regulation. It is important that, except one, none of these sno/scaRNAs were significantly affected when *hsrωRNAi* or *EP3037* is expressed under the *sev-GAL4* in normal Ras background (Fig. 5D). Interestingly, *sev-GAL4* driven *hsrωRNAi* or *EP3037* expression in normal Ras background affected some other sno/scaRNAs which were not affected by activated Ras expression (data not presented).

Of the 45 (7%) genes (encircled in white in Fig. 5B, and Fig. 5E) that were commonly up-regulated following *hsrωRNAi* or *EP3037* co-expression in *sevGAL4>Ras*^{V12} expressing eye discs compared to those of *sevGAL4>Ras*^{V12}, many were down-regulated or showed no change in *sevGAL4>Ras*^{V12} when compared with *sevGAL4>UAS-GFP* control eye discs. This group of 45 genes (Fig. 5B, E) included diverse genes, none of which seem to be directly involved in Ras signaling pathways. However, the *kuz* (*kuzbanian*) gene, encoding a metalloprotease, is expressed in developing ommatidia with roles in neuroblast fate determination, round-about (Robo) signaling and neuron formation (Coleman et al., 2010; Sotillos et al., 1997; Udolph et al., 2009). Therefore, this may be one potentially important gene which is up-regulated only when *hsrω* RNA levels are altered in activated Ras expressing eye discs, which as noted above showed increased numbers of R7 photoreceptors.

It is notable that most of the above 88 and 45 genes that showed common down- or up-regulation in *sevGAL4>Ras*^{V12} *hsrωRNAi* and *sevGAL4>Ras*^{V12} *EP3037* eye discs compared with *sevGAL4>Ras*^{V12}, appeared to be affected in opposite ways when the *hsrω* transcripts were down- or up-regulated in normal Ras background (columns 2 and 3 in Fig. 5C-E).

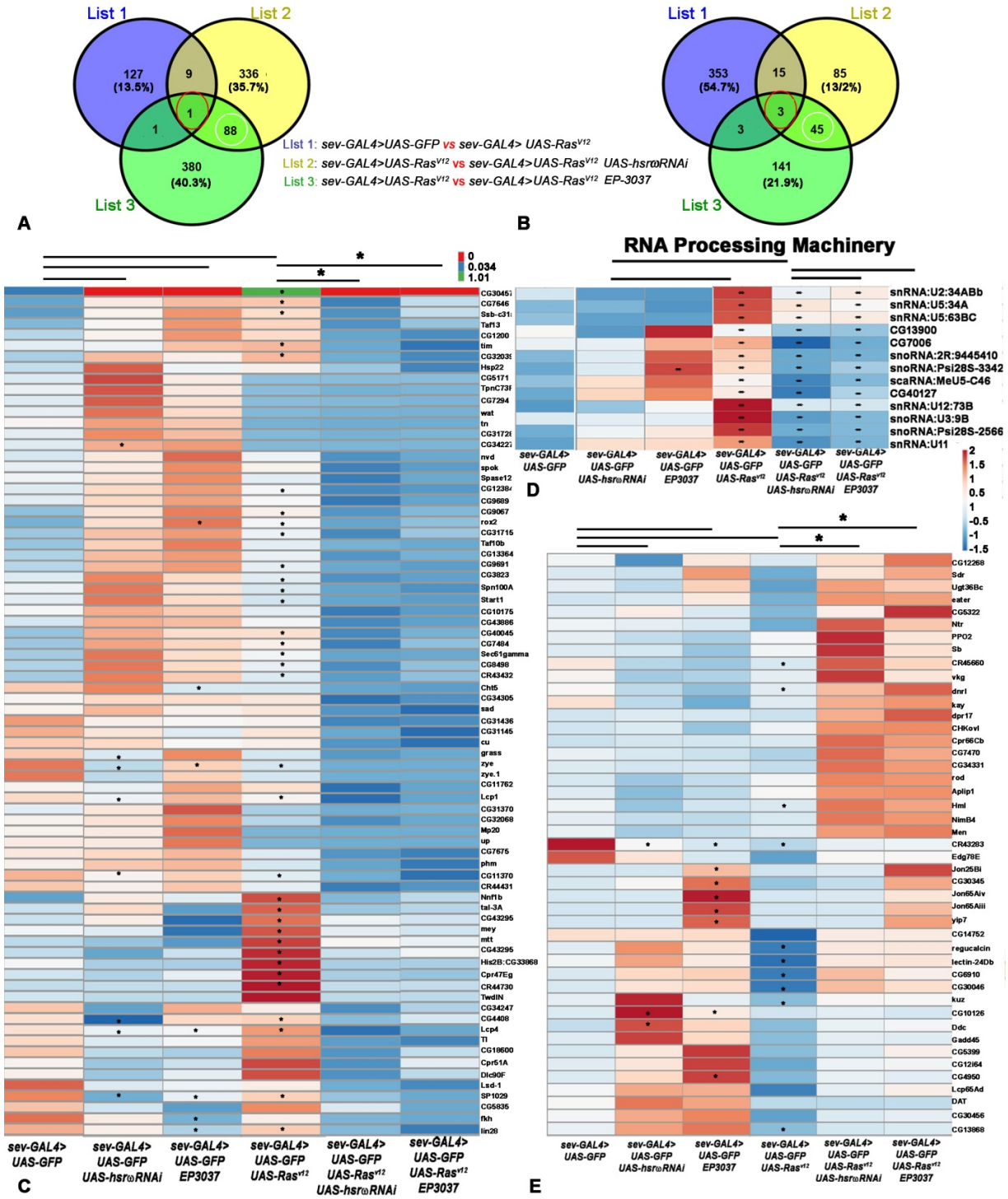

Fig. 5. Many transcripts, which were significantly up- or down-regulated following *sev-GAL4>*activated Ras expression, showed opposite changes that were similar irrespective of reduction or elevation of *hsrω* RNA levels. A-B Venn diagrams showing numbers of genes down- (**A**) or up- (**B**) regulated in third instar larval eye discs with either decrease (List 2) or

increase (List 3) in levels of *hsrω* RNA achieved by driving *UAS-hsrωRNAi* or *EP3037*, respectively, under *sev-GAL4* driver along with activated Ras expression as compared to control (*sev-GAL4>UAS-Ras*^{V12} vs *sev-GAL4>UAS-GFP*, List 1). Gene numbers in red circles in **A** and **B** denote genes commonly down- or up-regulated, respectively, in *sev-GAL4>UAS-hsrωRNAi UAS-Ras*^{V12}, *sev-GAL4>EP3037 UAS-Ras*^{V12} and *sev-GAL4>UAS-Ras*^{V12}. Numbers in white circles in **A** and **B** denote genes down- or up-regulated, respectively, in *sev-GAL4>UAS-hsrωRNAi* and *UAS-Ras*^{V12}*sev-GAL4> EP3037 UAS-Ras*^{V12} but not in *sev-GAL4>UAS-Ras*^{V12}. **C-D** Heat maps of FPKM values of different transcripts (noted on right of each row) of diverse GO terms (**C**) or RNA processing machinery (**D**) in different genotypes (noted at base of each column) which, compared to *sev-GAL4>UAS-GFP* (column 1), showed either no change or up-regulation in *sev-GAL4>*activated Ras expressing eye discs (column 4) but were significantly down-regulated in eye discs co-expressing *sev-GAL4>*activated Ras and altered *hsrω* RNA levels (columns 5 and 6). **E** Heat maps of FPKM values of genes which showed no change or down-regulation following activated Ras expression but were significantly up-regulated in eye discs expressing activated Ras and co-altered *hsrω* RNA levels. Heat maps of transcripts in *sev-GAL4>UAS-GFP* (column 1), *sev-GAL4>UAS-hsrωRNAi* (column 2) and *sev-GAL4>EP-3037* (column 3) are also shown in all cases for comparison. Asterisks indicate significant differences ($p \leq 0.05$) between the compared genotypes connected by horizontal bars on top; the Asterisk marks above horizontal lines connecting columns 4-5 and 4-6 in **C-E** indicate significant differences in all genes in the columns. Colour key for expression levels for the top row in **C** is shown above **C,** while that for all others is shown in middle right.

Finally, we examined genes that were differentially affected when *hsrωRNAi* or *EP3037* were co-expressed with activated Ras. These genes belonged to different pathways, but one group that appeared significant was that of positive and negative modulators of Ras signaling and photoreceptor differentiation (Fig. 6). The Gene ontology search results revealed that down-regulation of *hsrω* transcripts in activated Ras background enhanced levels of positive modulators Ras signaling in R7 cell fate determination (*phly, svp, rau* and *socs36E*) compared to only activated Ras expressing eye discs (Fig. 6). None of these four genes were up-regulated when *hsrω* transcripts were over-expressed in activated Ras background (Fig. 6). However, several negative regulators of Ras signal transduction pathway like *bru, klu, mesr4, cdep, epac,, nfat, ptp-er, cg43102, rhogap1a, rhogef2, spri, exn* etc were down regulated (Fig 6) in *sev-GAL4>Ras*^{V12} *EP3037* eye discs but not when *sev-GAL4>UAS-hsrωRNAi* was co-expressed with activated Ras. On the basis of David Bioinformatics GO term classification , genes like *bru, cdep, sos, pdz-gef, cg43102, rhogap1a, rhogef2, spri, exn* in this group are involved in Ras guanyl nucleotide exchange factor activity while the other genes like *klu, mesr4, nfat, ptp-er* affect small GTPase mediated signal transduction. Being negative-regulators, their down-regulation by co-expression of activated Ras and *EP3037* would lead to further enhanced Ras activity. It is interesting to note that in normal developmental Ras activity background, the *sev-GAL4* driven expression of *UAS-hsrωRNAi* or *EP3037* did not exert comparable differential effects on expression of these positive and negative modulators of Ras signaling since as shown

in columns 2 and 3 in Fig. 6, most of the affected genes were either not affected or were commonly down regulated when compared with *sev-GAL4>UAS-GFP* eye discs.

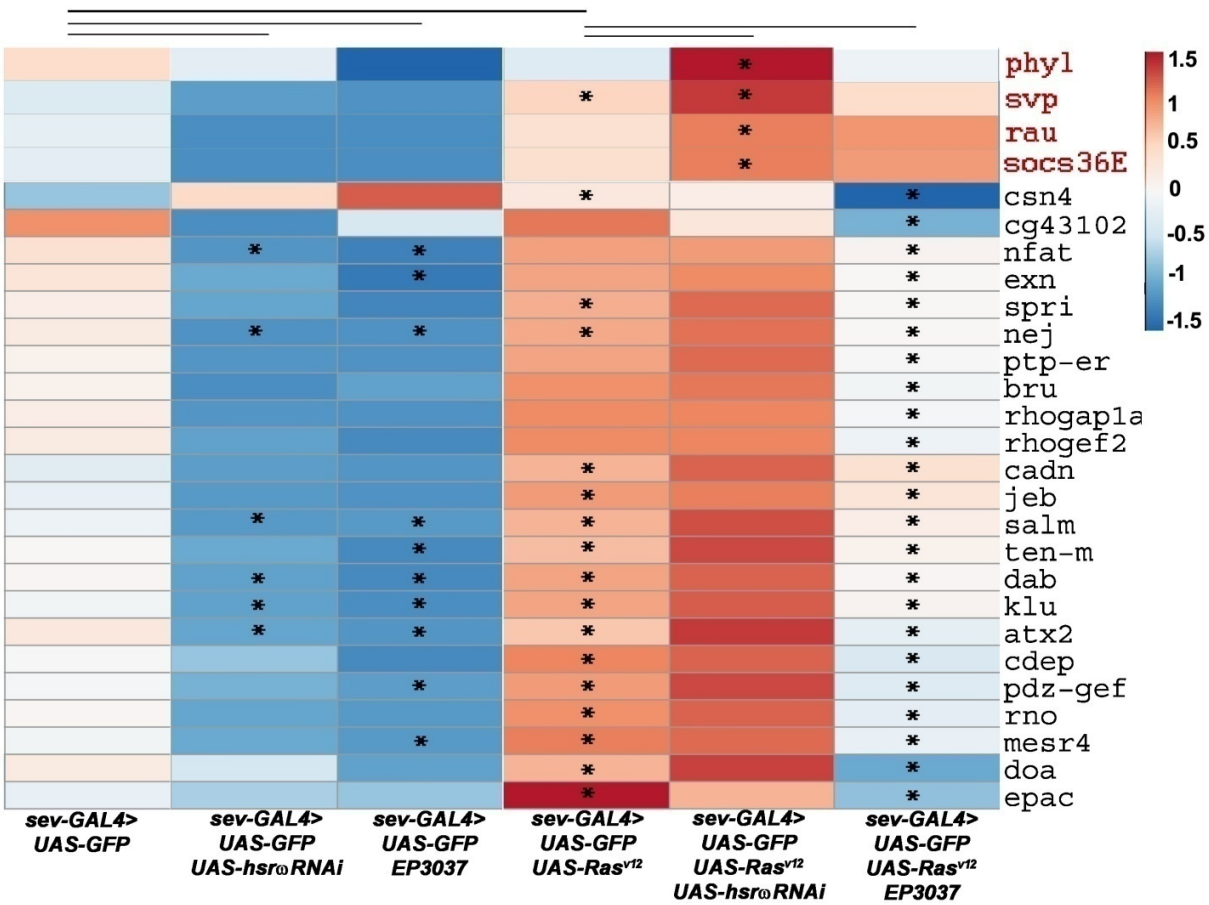

Fig. 6. Transcripts of positive Ras modulators are up-regulated while negative modulators are down-regulated upon down- and up-regulation, respectively, of *hsrω* transcripts in activated Ras expression background. Heat maps of FPKM values of transcripts involved in Ras signaling and photoreceptor differentiation (noted on right of each row) in different genotypes. The four genes potential positive modulators of Ras signaling which are up-regulated in *sev-GAL4>Ras^{V12} hsrωRNAi* are marked in red letters, Asterisks indicate significant differences ($p \leq 0.05$) between the compared genotypes connected by horizontal bars on top.

Alterations in *hsrω* RNA levels in activated Ras background affects activities of many transcription factor and RNA-binding protein genes

Since the above analysis did not show any significant changes in transcriptional activities of genes encoding components of the Ras signaling pathway, we argued that the elevated Ras signaling seen in eye discs co-expressing *sev-GAL4* driven activated Ras and altered levels of *hsrω* transcripts, may be due to changes in activities of other genes that affect transcription and post-transcriptional processing events for genes which may affect Ras signaling at post-

transcriptional levels. Therefore, we examined activities of genes coding for transcription factors (TF) and RNA-binding proteins (RBP) in different genotypes.

Out of ~1000 known TF (Rhee et al., 2014) in *Drosophila melanogaster*, 29 and 81 showed differential expression in *sev-GAL4>UAS-Ras^{V12} UAS-hsrωRNAi* and *sev-GAL4>UAS-Ras^{V12} UAS-EP3037*, respectively, when compared to *sev-GAL4>UAS-Ras^{V12}* (Fig. 7A, B). While 73 TF genes were uniquely down regulated in *sev-GAL4>UAS-Ras^{V12} UAS-EP3037*, only 9 were down-regulated in *sev-GAL4>UAS-Ras^{V12} UAS-hsrωRNAi* (Fig. 7A). On the other hand, compared to 14 TF genes being uniquely up-regulated in *sev-GAL4>UAS-Ras^{V12} UAS-hsrωRNAi*, up-regulation of *hsrω* transcripts in activated Ras background enhanced only 2 TF transcript levels (Fig. 7B). Thus a larger proportion of TF were down-regulated on over-expression of *hsrω* transcripts in activated Ras background whereas TF were more often up-regulated when *hsrω* transcripts were lowered in activated Ras background. The number of commonly down- or up- regulated TF was small, being 4 (*CG11762/ouijaboard*, *lin-28*, *forkhead*, *Ssb-c31a*) and 2 (*Ets21C* and *kay*), respectively (Fig. 7A, B). *CG11762* and *forkhead* genes are part of the ecdysone signaling pathway (Cao et al., 2007; Komura-Kawa et al., 2015). Ssb-c31a, as noted earlier, is reported to bind to Raf protein. The two TF, which were commonly up-regulated when *hsrω* RNAs were down- or up-regulated in active Ras expression background, are part of JNK signaling pathway. Gene Ontology search shows, as noted earlier (Fig. 6), that three of the many TF, viz., Klu, Mesr4, Nfat, which are uniquely down-regulated in *sev-GAL4>UAS-Ras^{V12} UAS*-EP3037 (Fig. 7F) are negative regulators of Ras signaling. On the other hand, the five of the 14 TF, viz., Svp, Peb, Gl, Ro and H, which are up-regulated in *sev-GAL4>UAS-Ras^{V12} UAS-hsrωRNAi* (Fig. 7 E) are involved in rhabdomere differentiation (Frankfort and Mardon, 2002; Kimmel et al., 1990; Kumar and Moses, 2000; Liang et al., 2016; Moses et al., 1989; Pickup et al., 2002)

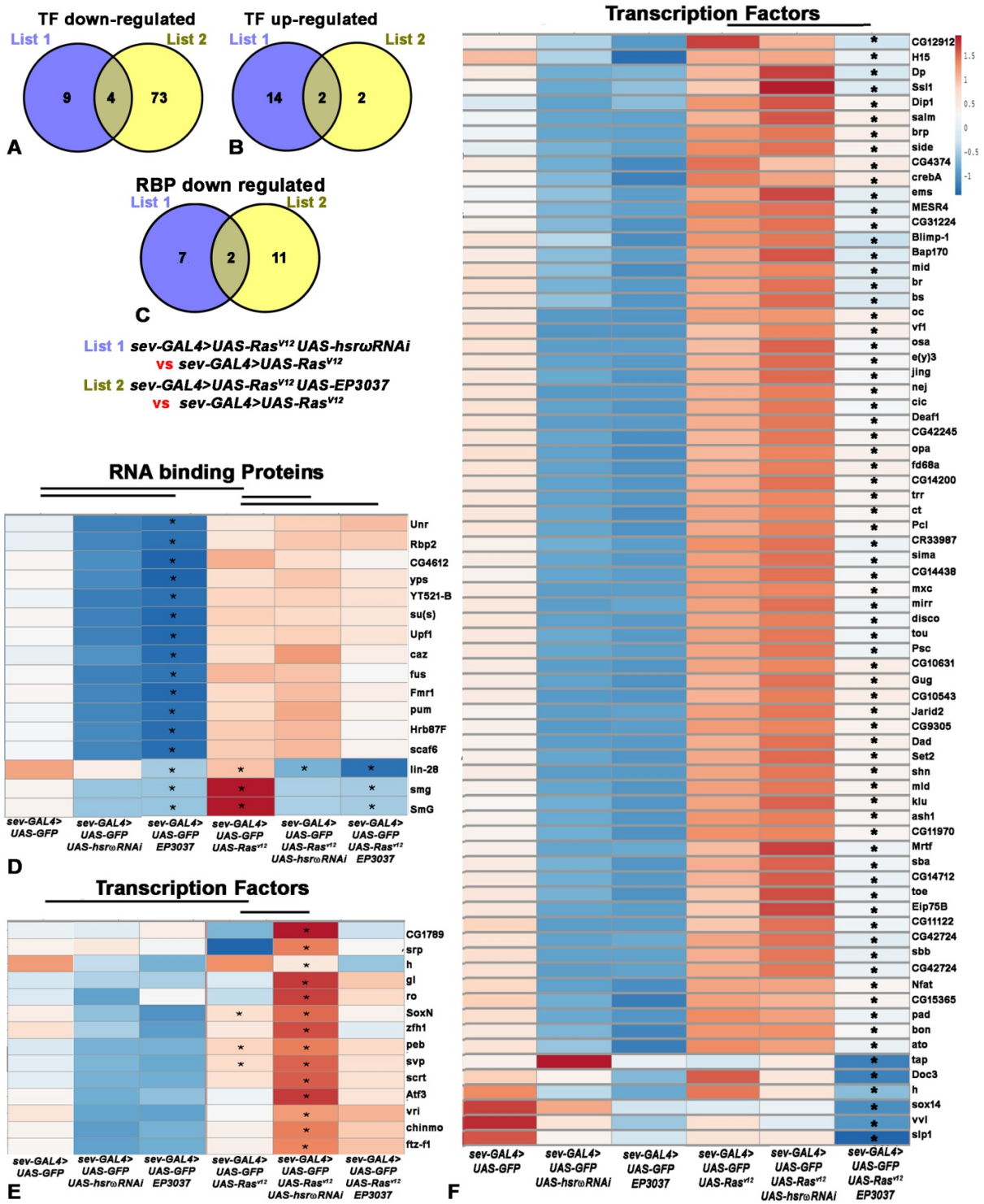

Fig. 7. Many RNA binding proteins and Transcription factor transcripts show varyingly different levels when *hsrω* RNA levels are reduced or elevated in activated Ras background than in normal Ras expression background. A-B Venn diagrams showing numbers of TF genes showing down- (**A**) or up-regulation (**B**) in third instar larval eye discs with either decrease (List1) or increase (List2) in levels of *hsrω* RNA levels in *sev-GAL4* driven activated Ras

expression background as compared to control eye discs (*sev-GAL4>UAS-Ras*^{V12}). **C** Venn diagrams showing numbers of RBP genes showing down-regulation following either decrease (List1) or increase (List 2) in levels of *hsrω* RNA levels activated Ras expression background, as compared to control eye discs (*sev-GAL4>UAS-Ras*^{V12}). **D** Heat maps of FPKM values of different transcripts (noted on right of each row) of RBP in different genotypes (noted at base of each column) which are significantly down-regulated in *sev-GAL4>EP3037* compared to *sev-GAL4>UAS-GFP*. **E-F** Heat maps of FPKM values of TF gene transcripts in different genotypes (noted at base of each column) which are either significantly up-regulated in activated Ras and lowered *hsrω* RNA levels when compared to only activated Ras expressing eye discs (**E**) or significantly down-regulated in activated Ras and enhanced *hsrω* RNA levels (**F**) Asterisks indicate significant differences ($p \leq 0.05$) between the compared genotypes (indicated by the horizontal lines at the top.

Comparison of levels of known RBP (RNA binding protein Database at http://rbpdb.ccbr.utoronto.ca) in eye discs expressing activated Ras alone or along with changes in *hsrω* RNA levels showed that 9 and 13 RBP-encoding genes were uniquely down-regulated when *hsrω* RNAs were down- or up-regulated, respectively, in activated Ras background when compared to only activated Ras expressing eye discs. Two genes, *CG7006* (a ribosome biogenesis factor NIP7-like) and *lin-28*, regulating maturation of let-7 microRNA (Moss and Tang, 2003; Newman et al., 2008; Stratoulias et al., 2014; Viswanathan et al., 2008), were commonly down-regulated when *hsrω* transcripts were altered in activated Ras background (Fig 7C). Only one gene (*cpo*) was uniquely down-regulated in *sev-GAL4>UAS-Ras*^{V12} *UAS-hsrωRNAi*. Likewise only one gene (*fne*) was up-regulated in *sev-GAL4>UAS-Ras*^{V12} *EP-3037* eye discs. Interestingly, none of the known omega speckle associated RBPs showed any change at transcript level in any of the three activated Ras genotypes examined here.

Discussion

Present study was initiated to examine interactions between long non-coding *hsrω* RNAs and Ras signaling cascade. Although, some studies have implicated oncogenic Ras to influence expression of several long non coding transcripts (Jiang et al., 2017; Jinesh et al., 2018; Kotake et al., 2016; Rotblat et al., 2011; Zhang et al., 2017b), regulation of Ras signaling cascade by lncRNAs is not yet known. While an earlier study from our lab showed that a *ras* mutant allele enhanced *hsrω*-null phenotype (Ray and Lakhotia, 1998), the present study shows that alterations in levels of *hsrω* transcripts exaggerate phenotypes that follow the *sev-GAL4* driven expression of activated Ras in developing eye discs of *Drosophila* larvae. The *UAS-Ras*^{V12} transgene (Karim et al., 1996) has been widely used for examining consequence of ectopic expression of ligand-independent activated Ras. Its expression in developing eye discs under the *sev-GAL4* driver is known to disrupt ommatidial arrays because of recruitment of additional cells to R7 photoreceptor path (Karim et al., 1996). Our results clearly show that reduced as well as over-expression of *hsrω* transcripts in activated Ras background significantly enhanced the number of R7 photoreceptor per ommatidium unit. As revealed by detection of pMAPK, Yan

and activated Ras associated RafRBDFLAG in eye discs, the increase in R7 photoreceptors was distinctly correlated with the enhanced Ras activity levels in *sev-GAL4>hsrωRNAi UAS-Ras*^{V12} as well as *sev-GAL4>EP3037 UAS-Ras*^{V12} genotypes.

The similar enhancing effect of down- or up-regulation of *hsrω* transcripts on Ras signaling appears contrary to the common logic. In order to rule out non-specific effects, we also used a different RNAi transgene (*UAS-pUHEx2A*, R. R. Sahu and S. C. Lakhotia, unpublished) that targets the exon 2 of *hsrω* transcripts, a region common to all the different transcripts produced by this gene to down-regulate *hsrω* transcripts (Lakhotia, 2017a), and another EP line, *EP93D* (Mallik and Lakhotia, 2009) to over-express *hsrω*. In each case, the Ras signaling was comparably enhanced when these were co-expressed with *sev-GAL4>UAS-Ras*^{V12}. The enhanced Ras activity, reflected in increased R7 photoreceptors and greater roughening of eyes following expression of *sev-GAL4>UAS-Ras*^{V12} in background of *hsrω*^{66}, a near-null allele of *hsrω*, also confirms enhanced Ras activity when *hsrω* transcript levels are reduced. Together, these results confirm that the similar end-result of down- or up-regulation of *hsrω* transcripts on Ras signaling in high activated Ras background reflects specific consequences of the complex interactions between ectopically expressed activated Ras and altered levels of *hsrω* lncRNAs. It may be mentioned that these same lines have been used in several earlier studies (Lakhotia et al., 2012; Mallik and Lakhotia, 2009; Mallik and Lakhotia, 2010; Mallik and Lakhotia, 2011; Onorati et al., 2011; Piccolo et al., 2018; Piccolo and Yamaguchi, 2017; Singh and Lakhotia, 2015) without any indication of nonspecific effects, although similar as well as dissimilar end-phenotypes were seen following down- or up-regulation of the *hsrω* gene expression.

Since the levels of *Ras* or *Ras*^{V12} transcripts were not affected by the down- or up-regulation of *hsrω* transcripts in *sev-GAL4>Ras*^{V12} *UAS-hsrωRNAi* or *sev-GAL4>Ras*^{V12} *EP3037* eye discs, respectively, while the levels of total as well as activated Ras protein were significantly elevated in both cases (Fig. 3), we believe that altered levels of *hsrω* transcripts in high activated Ras background modulate Ras activity through post-transcriptional events.

It is significant that down- or up-regulation of *hsrω* transcripts in wild type Ras background does not affect ommatidial differentiation since the eyes in *sev-GAL4>UAS-hsrωRNAi* or *sev-GAL4>EP3037* flies are normal with no extra rhabdomere (Mallik and Lakhotia, 2011). Immunostaining for Ras and RafRBDFLAG in *sev-GAL4>UAS-GFP* or *sevGAL4>UAS-hsrωRNAi* or *sev-GAL4>EP3037* eye discs also did not show any difference between them (not shown). Apparently, ectopic over-expression of activated Ras makes the cells detectably sensitive to alterations in *hsrω* transcript levels. This is also reflected in the many different, and sometimes opposing, effects on the transcriptomes of eye discs expressing activated Ras in normal and altered levels of *hsrω* transcripts or vice-versa. Such changes have implications for a role of the *hsrω* transcripts in modulating consequences that follow an aberrant Ras activity during development.

The significant increase in Ras activity in *sev-GAL4>Ras^{V12} UAS-hsrωRNAi* or *sev-GAL4> Ras^{V12} EP3037* eye discs seems to be due to modulation of activities of other regulators of the Ras signaling cascade. Our data show (Fig. 6) that *sev-GAL4>Ras^{V12} UAS-hsrωRNAi* eye discs have significantly elevated levels of transcripts of some of the genes (*phly, svp, rau* and *socs36E*) whose products have been identified as positive modulators of Ras activity in R7 differentiation. The *phly* encodes a nuclear receptor, which acts downstream of Ras and is responsible for R7 fate determination (Chang et al., 1995). The Svp is an orphan nuclear receptor responsible for transforming cone cells to R7 photoreceptor cells in conjunction with activated Ras (Begemann et al., 1995; Kramer et al., 1995). Svp expression is also known to increase Rau expression, which sustains RTK signaling downstream of Htl or Egfr via the two Ras association (RA) domains, which have a binding preference for GTP-loaded Ras to maintain its activity via a positive feedback loop (Sieglitz et al., 2013). The Socs36E too can amplify Ras/MAPK signaling in precursors of R7 cells (Almudi et al., 2010). Thus, up-regulation of these genes in eye discs with decreased *hsrω* RNA levels in activated Ras expression background correlates with the observed greater increase in R7 photoreceptors than when activated Ras is expressed alone. None of these positive modulators showed enhanced activity in *sev-GAL4>Ras^{V12}UAS-EP3037* eye discs but transcripts of another set of genes (*bru, klu, mesr4, cdep, epac, nfat, ptp-er, cg43102, rhogap1a, rhogef2, spri, exn* etc), which can act as negative modulators of the Ras cascade, were down regulated (Fig. 6). Some of these genes, viz., *bru, cdep, sos, pdz-gef, cg43102, rhogap1a, rhogef2, spri, exn*, are involved in Ras guanyl nucleotide exchange factor activity (Jékely et al., 2005; Lee et al., 2002; Yan and Perrimon, 2015) while others like *klu, mesr4, nfat, ptp-er* are negative regulators of small GTPase mediated signal transduction (Ashton-Beaucage et al., 2014; Brachmann and Cagan, 2003; Huang and Rubin, 2000). Down-regulation of these negative modulators would result in elevated Ras activity and signaling. In either cases, therefore, the net result would be up-regulation of Ras activity as actually seen in *sev-GAL4>UAS-hsrωRNAiUAS-Ras^{V12}and sev-GAL4>UAS-EP3037 UAS-Ras^{V12}* eye discs. The action of positive regulators can be expected to be stronger than that of the negative modulators and this may contribute to the greater enhancement in Ras signaling in *sev-GAL4>Ras^{V12}UAS-hsrωRNAi* than in *sev-GAL4>Ras^{V12}UAS-EP3037* eye discs. The observed up-regulation of Ssb-c31a in *sev-GAL4>Ras^{V12}UAS-hsrωRNAi* as well as *sev-GAL4>Ras^{V12}UAS-EP3037* eye discs can also contribute to the elevated Ras signaling through its binding with Raf (Friedman et al., 2011).

Since the *hsrω* transcripts are not known to bind with gene promoters or other RNAs, the changes in activities of the above noted negative and positive modulators of Ras signaling appears to be related to changes in activities of the diverse proteins that associate with these lncRNAs. Of the multiple transcripts produced by *hsrω* gene (Lakhotia, 2011; Lakhotia, 2017b), the >10kb nucleus-limited transcripts are essential for biogenesis of the omega speckles and consequently for the dynamic movement of the omega speckle associated proteins like various hnRNPs, certain other RNA binding proteins and some nuclear matrix proteins etc (Lakhotia et al., 2012; Mallik and Lakhotia, 2011; Piccolo et al., 2018; Piccolo and Yamaguchi, 2017; Singh and Lakhotia, 2015). The GAL4 induced expression of *hsrωRNAi* transgene or of the *EP3037*

allele of *hsrω* is known to primarily lower and elevate, respectively, levels of the larger and nucleus-limited transcripts of *hsrω* gene (Mallik and Lakhotia, 2009) (Mallik and Lakhotia, 2011). Either of these conditions disrupt the dynamics of omega speckles and their associated proteins involved in RNA processing (Lakhotia et al., 2012; Piccolo et al., 2018; Piccolo and Yamaguchi, 2017; Singh and Lakhotia, 2015). In view of the multiple and diverse roles played by the omega speckle associated proteins in gene expression and post-transcriptional processing/stability of diverse RNAs, the lncRNAs like those produced by *hsrω* gene have been considered to act as hubs in complex cellular networks (Arya et al., 2007; Lakhotia, 2012; Lakhotia, 2016). The disrupted dynamics of several important members of RNA processing machineries may indeed be responsible for the rather unexpected observed commonality in enhancing the Ras signaling cascade when the *hsrω* nuclear transcript levels were down or up-regulated in the background of ectopic expression of activated Ras. This is further supported by the transcriptomic data which shows that up or down regulation of *hsrω* RNAs results in many similar changes in eye discs of developing third instar larvae.

The Caz/dFus protein, a hnRNP family member, physically and genetically interacts with *hsrω* nuclear transcripts so that following down regulation of *hsrω* nuclear transcripts through RNAi, this protein moves out of nucleus to cytoplasm (Piccolo and Yamaguchi, 2017). Our transcriptome analysis shows that *EP3037* expression reduces levels of *Caz/dFus* transcripts. A bioinformatic search (data not presented) for potential binding of Caz/dFus protein with transcripts of the positive and negative modulators of Ras signaling, whose transcript levels were found to be enhanced or reduced (Fig.6) when *hsrω* nuclear transcript levels were altered in activated Ras background, revealed that many of these transcripts indeed carry binding sites for Caz/dFus. Significantly, another hnRNP family member which interacts with omega speckles and with Caz/dFus protein is the TDP-43 (dTBPH) (Appocher et al., 2017; Coyne et al., 2015; Piccolo et al., 2018; Romano et al., 2014). The Caz/dFus and TDP-43 also interact with Fmr1 RBP, and the three of them are involved in complex interactions with variable consequences for processing, translatability and stability of diverse mRNAs (Appocher et al., 2017; Coyne et al., 2015; Piccolo et al., 2018; Piccolo and Yamaguchi, 2017; Romano et al., 2014). It is significant that like the many other RBPs, each of these three genes produce multiple transcripts and multiple protein products (http://Flybase.org). Thus depending upon the context, the *hsrω* interacting proteins can affect different sets of transcripts differently and affect their abundance, translatability and turnover etc. Although a role of any of these proteins in Ras signaling in *Drosophila* has not yet been shown, many genes of the Ras signal transduction pathway were found to be differentially expressed in Caz/dFus depleted mouse cell lines (Honda et al., 2014).

The *let-7* miRNA binds with the 3'UTR of *Ras* transcripts and its levels are lowered by high levels of Ras protein while *let-7* over expression lowers Ras protein levels (Jinesh et al., 2018; Johnson et al., 2005; Wang et al., 2013). Interestingly, the pre as well as mature *let-7* miRNA also bind to *TDP-43* and *Fmr1* transcripts (Buratti et al., 2010; Yang et al., 2009). TDP-43 knock down down-regulates *let-7b* miRNA (Buratti et al., 2010; Chen et al., 2017; Kumar et al.,

2016). It is possible that the TDP-43's movement away from nucleus to cytoplasm in *hsrω* RNA depleted condition (Piccolo et al., 2018) can reduce the availability of functional *let-7*, resulting in further enhanced Ras activity. Another protein which regulates maturation of *let-7* miRNA is Lin-28 (Moss and Tang, 2003; Newman et al., 2008; Stratoulias et al., 2014; Viswanathan et al., 2008). Therefore, the observed up-regulation of *Lin-28* transcripts in *sev-GAL4>Ras^{V12}UAS-hsrωRNAi* as well as *sev-GAL4>Ras^{V12}UAS-EP3037* eye discs too can affect *let-7* miRNA activity and consequently, Ras protein levels.

Our RNA-seq data further showed that *hsrω* RNA levels also directly or indirectly affect other small ncRNAs since certain snoRNAs, snRNAs and scaRNAs, which are highly up regulated in activated Ras background were significantly down-regulated when the *hsrω* transcripts were either up- or down-regulated. Changes in levels of the small ncRNAs may also contribute to the enhanced Ras signaling in these genotypes. Although the most widely appreciated function of snoRNAs is their roles in maturation of rRNAs (Dieci et al., 2009; Dragon et al., 2006; Henras et al., 2015; Sloan et al., 2017), they have other regulatory roles as well, including modifications of some snRNAs (Dupuis-Sandoval et al., 2015; Falaleeva and Stamm, 2013; McMahon et al., 2015). The Cajal body associated scaRNAs are essential for proper functioning and maturation of snRNAs, which in turn are critical for appropriate processing of mRNAs (Darzacq et al., 2002; Deryusheva and Gall, 2009; Kiss et al., 2002; Kiss, 2004; Richard et al., 2003). The cumulative consequences of alterations in these diverse small RNAs (sca, sn and snoRNAs) on different genes' transcriptional and translational activities can be very extensive. It would be interesting to examine the possibility that some of these small ncRNAs are part of an auto-inhibitory loop in Ras signaling such that their down regulation results in further enhanced Ras activation. Loss of two snoRNAs, SNORD50A and SNORD50B, in human cells has been shown to be associated with increased levels of active K-Ras leading to hyperactivated Ras/ERK signaling (Siprashvili et al., 2016). Homologs of these two snoRNAs are not yet identified in flies.

Several ribosomal protein genes show reduced expression following activation of the Ras pathway (Friedman et al., 2011). It remains to be examined if the observed further reduction in some of the ribosomal proteins gene transcripts in *sev-GAL4>Ras^{V12} UAS-hsrωRNAi* eye discs has any effect on the very high Ras activity in these eye discs. Likewise, further studies are needed to identify roles, if any, of altered expression of genes like *CG13900*, *dlc90F* and *kuz* in further elevating the Ras signaling in the experimental genotypes.

Our study clearly shows complex interaction between Ras signaling and the *hsrω* lncRNAs. The normal developmental Ras activity was not affected, at least in the eye discs, by alterations in abundance of these RNAs, although many other transcripts were affected. On the other hand, when Ras activity was ectopically elevated, less or more abundance of *hsrω* lncRNAs not only enhanced Ras signaling but also affected different sets of genes than in eye disc cells with normal Ras activity. Such context-dependent roles of the *hsrω* lncRNAs agree with their action as hub in cellular network.

An interesting finding of the present study was the marked increase in cell non-autonomous Ras signaling. The *sev-GAL4* driven activated Ras as well as RafRBDFLAG transgenes were expressed only in a specific subset of cells in the eye discs. Therefore, the presence of RafRBDFLAG bound Ras in adjacent non *sev-GAL4* expressing cells is unexpected and suggests that the activated Ras-Raf complex can move out of the source cells to neighbouring cells. The great reduction in Yan expression even in MF region, where the *sev-GAL4* driver is not at all expressed, when activated Ras expression is accompanied by altered *hsrω* transcript levels also provides strong evidence for existence of cell non-autonomous Ras signaling. Finally, the much greater number of R7 cells in discs that co-expressed *sev-GAL4* driven activated Ras and altered levels of *hsrω* transcripts also shows that neighbouring cells that did not express *sev-GAL4* also got recruited to the R7 pathway because of the active Ras complex moving out of the *sev-GAL4* expressing cells. Although several studies (Enomoto et al., 2015; Parry and Sundaram, 2014; Takino et al., 2014; Uhlirova et al., 2005; Yan et al., 2009) have indicated cell non autonomous Ras signaling, only one study reported transfer of GFP tagged H-Ras to T cells from the antigen-presenting cells (Goldstein et al., 2014). Our results also strongly indicate movement of the activated Ras complex across to neighbouring cells.

Our findings, besides highlighting roles of non coding part of the genome in modulating important signaling pathway like Ras, also unravel new insights into the working of Ras signaling cascade itself. Our finding of a non cell autonomous spread of Ras signaling and that a long non-coding RNA can further increase the already elevated Ras activity are significant in view of the fact that activated Ras/Raf mutations are implicated in diverse malignancies. Although some earlier studies indicated roles of certain lncRNAs in ERK/MAPK activation in cancer (Kotake et al., 2016; Xu et al., 2014; Zhang et al., 2017a), their roles in enhancing Ras activity itself has not been known. Future studies on interactions between the diverse small nc and lncRNAs and signaling pathways like Ras are expected to unravel new dimensions of cellular networks that regulate and determine basic biological processes of cell proliferation, differentiation and death on one hand, and roles in cancer on the other.

Materials and Methods

Fly stocks

All fly stocks and crosses were maintained on standard agar-maize powder-yeast and sugar food at 24 ± 1^{0}C. The following stocks were obtained from the Bloomington Stock Centre (USA): w^{1118}*; sev-GAL4;* + (no. 5793; Bailey 1999), w^{1118}*; UAS-GFP* (no. 1521), w^{1118}*; UAS-Ras*^{V12} (no. 4847). For targeted (Brand and Perrimon, 1993) down-regulation of the hsrω transcripts, *UAS-hsrω-RNAi*^{3} transgenic line (Mallik and Lakhotia, 2009) was used; in some cases another RNAi transgene, *UAS-pUHEx2ARNAi*, which targets the exon 2 region of the *hsrω* gene (R. R. Sahu and S. C. Lakhotia, unpublished) was also used. Up regulation of the *hsrω* was achieved by expressing *EP3037* allele, or in a few cases the *EP93D* allele, under the *sev-GAL4* driver (Mallik and Lakhotia, 2009). The *UAS-hsrω-RNAi*^{3} and the *EP3037* lines are referred to in the text as

UAS-hsrωRNAi and *EP3037*, respectively. For *hsrω*-null condition, the *w: hsrω^{66}/hsrω^{66}* stock (Johnson et al., 2011) was used. The *UAS-RafRBDFLAG* stock (Freeman et al., 2010) was provided by Dr. S. Sanyal (Emory University, USA). Using these stocks, appropriate crosses were made to finally obtain progenies of the following genotypes:

a) *w^{1118};* sev-GAL4 *UAS-GFP/UAS-GFP; dco^{2} e/+*

b) *w^{1118}; sev-GAL4 UAS-GFP/UAS-GFP; dco^{2} e/UAS- Ras^{V12}*

c) *w^{1118}; sev-GAL4 UAS-GFP/UAS-GFP; UAS-hsrωRNAi/UAS-Ras^{V12}*

d) *w^{1118}; sev-GAL4 UAS-GFP/UAS-GFP; EP3037/UAS-Ras^{V12}*

e) *w^{1118}; sev-GAL4 UAS-GFP/UAS-GFP; EP93D/UAS-Ras^{V12}*

f) *w^{1118}; sev-GAL4 UAS-GFP/UAS-pUHEx2ARNAi; +/UAS-Ras^{V12}*

g) *w^{1118}; sev-GAL4 UAS-GFP/UAS-GFP; hsrω^{66}/hsrω^{66} UAS-Ras^{V12}*

h) *w^{1118};* sev-GAL4 *UAS-GFP/UAS-RafRBDFLAG; dco^{2} e/+*

i) *w^{1118}; sev-GAL4 UAS-GFP/UAS-RafRBDFLAG; dco^{2} e/UAS-Ras^{V12}*

j) *w^{1118}; sev-GAL4 UAS-GFP/UAS-RafRBDFLAG; UAS-hsrωRNAi/UAS-Ras^{V12}*

k) *w^{1118}; sev-GAL4 UAS-GFP/UAS-RafRBDFLAG; EP3037/UAS-Ras^{V12}*

The *w^{1118}, dco^{2}* and *e* markers are not mentioned while writing genotypes in Results.

Lethality Assay

For lethality assay, freshly hatched 1st instar larvae of *sev-GAL4>UAS-GFP*, *sev-GAL4>Ras^{V12}*, *sev-GAL4>UAS-Ras^{V12}UAS-hsrωRNAi* and *sev-GAL4>UAS-Ras^{V12}EP3037* were collected during one hour interval and gently transferred to food vials containing regular food and reared at 24±1°C or at 18±1°C. The total numbers of larvae that pupated and subsequently emerged as flies were counted for at least three replicates of each experimental condition and/or genotypes.

Photomicrography of adult eyes

For examining the external morphology of adult eyes, flies of the desired genotypes were etherized and their eyes photographed using a Sony Digital Camera (DSC-75) attached to a Zeiss Stemi SV6 stereobinocular microscope or using Nikon Digital Sight DS-Fi2 camera mounted on Nikon SMZ800N stereobinocular microscope.

Nail polish imprints

The flies to be examined for organization of ommatidial arrays were anaesthetized and decapitated with needle and the decapitated head was briefly dipped in a drop of transparent nail polish placed on a slide. It was allowed to dry at RT for 5-10 min after which the dried layer of nail polish was carefully separated from the eye tissue with the help of fine dissecting needles and carefully placed on another clean glass slide with the imprint side facing up and flattened by gently placing a cover slip over it as described earlier (Arya and Lakhotia, 2006). The eye imprints were examined using 20X DIC optics.

Whole organ immunostaining

Eye discs from actively migrating late third instar larvae of desired genotypes were dissected out in PSS and immediately fixed in freshly prepared 3.7%paraformaldehyde in PBS for 20 min and processed for immunostaining as described earlier (Prasanth et al., 2000). The following primary antibodies were used: rat monoclonal anti-Elav (DSHB, 7E8A10, 1:100), rabbit monoclonal anti-Ras (27H5, Cell signaling, 1:50), mouse anti-FLAG M2 (Sigma-Aldrich, India, 1:50), rabbit p-MAPK (Phospho-p44/42 MAPK (Thr202, Tyr204), D13.14.4E, Cell signaling, 1:200), mouse anti-Yan (8B12H9, Developmental Studies Hybridoma Bank, Iowa, 1:100) and guinea pig anti-Runt, a gift by Dr. K. Vijaya Raghavan, India, (Kosman et al., 1998) at 1:200 dilution. Appropriate secondary antibodies conjugated either with Cy3 (1:200, Sigma-Aldrich, India) or Alexa Fluor 633 (1:200; Molecular Probes, USA) or Alexa Fluor 546 (1:200; Molecular Probes, USA) were used to detect the given primary antibodies. Chromatin was counterstained with DAPI (4', 6-diamidino-2-phenylindole dihydrochloride, 1µg/ml). Tissues were mounted in DABCO antifade mountant for confocal microscopy with Zeiss LSM Meta 510 using Plan-Apo 40X (1.3-NA) or 63X (1.4-NA) oil immersion objectives. Quantitative estimates of proteins in different regions of eye discs and co-localization were obtained, when required, with the help of Histo option of the Zeiss LSM Meta 510 software. All images were assembled using the Adobe Photoshop 7.0 software.

RNA isolation and Reverse Transcription-PCR

For semi-quantitative RT-PCR and qRT-PCR analyses, total RNA was isolated from eye imaginal discs of healthy wandering third instar larvae of the desired genotypes using Trizol reagent following the manufacturer's (Sigma-Aldrich, India) recommended protocol. RNA pellets were resuspended in nuclease-free water and quantity of RNA was estimated spectrophotometrically. The RNA samples (1µg) were incubated with 2U of RNase free DNaseI (MBI Fermentas, USA) for 30 min at 37°C to remove any residual DNA. First strand cDNA was synthesized from 1-2 µg of total RNA as described earlier (Mallik and Lakhotia, 2009). The prepared cDNAs were subjected to semi quantitative RT-PCR or real time PCR using forward and reverse primer pairs (see Supplementary Table S1). Real time qPCR was performed using 5µl qPCR Master Mix (SYBR Green, Thermo Scientific), 2 picomol/µl of each primer per reaction in 10 µl of final volume in an ABI 7500 Real time PCR machine.

The PCR amplification reactions were carried out in a final volume of 25 µl containing cDNA (50 ng), 25 pM each of the two specified primers, 200 µM of each dNTPs (Sigma Aldrich, USA) and 1.5U of *Taq* DNA Polymerase (Geneaid, Bangalore). The cycling parameters for specific transcripts are given in Supplementary Table S**2.** 15 µl of the PCR products were loaded on a 2% agarose gel to check for amplification along with a 50bp DNA ladder as a molecular marker.

Next Generation RNA sequencing

Total RNA was isolated from 30 pairs of eye discs of *sev-GAL4>UAS-GFP*, *sev-GAL4>UAS-hsrωRNAi*, *sev-GAL4>EP3037*, *sev-GAL4>UAS-Ras^{V12}*, *sev-GAL4>UAS-hsrωRNAi UAS-Ras^{V12}* and *sev-GAL4>EP3037 UAS-Ras^{V12}* late third instar larvae using Trizol (Invitrogen, USA) reagent as per manufacture's protocol. 1µg of the isolated RNA was subjected to DNAse treatment using 2U of TurboTM DNAse (Ambion, Applied Biosystem) enzyme for 30 min at 37°C. The reaction was stopped using 15mM EDTA followed by incubation at 65°C for 5-7 min and purification on RNAeasy column (Qiagen). The purified RNA samples were processed for preparations of cDNA libraries using the TruSeq Stranded Total RNA Ribo-Zero H/M/R (Illumina) and sequenced on HiSeq-2500 platform (Illumina) using 50bp pair-end reads, 12 samples per lane and each sample run across 2 lanes. This resulted in a sequencing depth of ~20 million reads. Biological triplicate samples were sequenced in each case. The resulting sequencing FastQ files were mapped to the *Drosophila* genome (dm6) using Tophat with Bowtie. The aligned SAM/BAM file for each was processed for guided transcript assembly using Cufflink 2.1.1 and gene counts were determined. Mapped reads were assembled using Cufflinks. Transcripts from all samples were subjected to Cuffmerge to get final transcriptome assembly across samples. In order to ascertain differential expression of gene transcripts between different samples, Cuffdiff 2.1.1 was used (Trapnell et al., 2012). A P-value <0.05 was taken to indicate significantly differentially expressing genes between different groups compared. Genes differentially expressed between experimental and control genotypes were categorized into various GO terms using DAVID bioinformatics Resources 6.8 (Huang et al., 2009) https://david.ncifcrf.gov for gene ontology search. In order to find out distribution of differentially expressing genes into various groups, Venn diagrams and Heat maps were prepared using the Venny2.1 and ClustVis softwares, respectively (Metsalu and Vilo, 2015).

The RNA sequence data files, showing pair wise comparisons of gene expression levels for different genotypes are presented in Supplementary Table S3

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Competing interests

Authors declare no conflicting interests

Author contributions

MR and SCL planned experiments, analyzed results and wrote the manuscript. MR carried out the experimental work and collected data.

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Data availability

The NGS data for RNA-sequencing in activated Ras expressing genotypes have been deposited at GEO (http://www.ncbi.nlm.nih.gov/geo/) with accession no. GSE107529. RNA-seq data for eye discs with *sev-GAL4* driven altered levels of *hsrω* transcripts in normal Ras background have been deposited at GEO; accession number for this submission is awaited.

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